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TITLE: Targeting the Nociceptin/Orphanin FQ Receptor for Scleroderma Therapy

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14. ABSTRACT
Scleroderma is a debilitating disease of the connective tissue that affects about 75,000–100,000 individuals in the United States and accounts for an estimated $400 million in total annual medical costs. There is no treatment that stops or cures scleroderma: novel therapies are therefore urgently needed. The overall objective of this proposal is to demonstrate that small molecule compounds that target receptor-ligand pair NOP receptor–Nociceptin/Orphanin FQ can limit the damage and malfunctions that occur in the immune system and in blood vessels during scleroderma. We anticipate that our approach will demonstrate that a single drug can limit inflammation, heal blood vessel damage, and increase blood flow.

15. SUBJECT TERMS
Nociceptin/orphanin FQ (N/OFQ), N/OFQ peptide receptor (NOPR), vascular endothelial cells, macrophages, partial agonist, scleroderma, fibrosis, vasculopathy, autoimmunity.

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1. **INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

This proposal addresses new approaches to treat scleroderma. The overall objective is to demonstrate that small molecules that target nociception/orphanin N/OFQ peptide receptor NOPR can limit the damage and malfunctions that occur in the immune system and in blood vessels during scleroderma. Our approach uses a combination of targeted in vitro cell-based assays and a preclinical rodent model of scleroderma to evaluate NOPR partial agonists for efficacy.

2. **KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

Nociceptin/orphanin FQ (N/OFQ), N/OFQ peptide receptor (NOPR), vascular endothelial cells, macrophages, partial agonist, scleroderma, fibrosis, vasculopathy, autoimmunity.

3. **ACCOMPLISHMENTS:** The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Specific Aim 1 **To test the hypothesis that distinct partial agonists for NOPR limit macrophage migration and vasculopathy.**	**Target Date**	**Actual Completion Date** *or →*	**Percentage Completion at Time of Final Report**
Major Task 1: Administrative tasks and experimental preparation	Months 1-6		100%
Subtask 1. Obtain local IACUC and ACURO approval for the animal studies	1-2	-1	
Subtask 2. Obtain and expand SVEC4-10 mouse cell line (ATCC)	1-2	2	
Subtask 3. Obtain NOPR-/- animals and establish breeding colony	1-6	14	
Milestone(s): Local IACUC and ACURO Approval	-1	-1	
Major Task 2: To test NOPR partial agonists for activity in three in vitro assays: endothelial cell wound healing, macrophage chemotaxis, and blood vessel tension (aortic ring assay)	3-12		66%

Subtask 1. Determine optimal NOPR agonist (N/OFQ) and antagonist (JTC-801) concentrations for the three proposed in vitro assays: scratch wound assay, macrophage chemotaxis using transwell inserts, and blood vessel tension using an aortic ring assay. In the scratch wound assay, a pipet tip is used to scratch or wound an endothelial monolayer, and the 'time-to-wound closure' is monitored over the next 24 hr by microscopy. In the macrophage chemotaxis assay, cells are loaded into the top well of a transwell insert and exposed to chemoattractants in the bottom well; the number of migrating cells is quantified by flow cytometry. In the aortic ring assay, freshly isolated thoracic aorta rings will be harvested and mounted in a small-vessel myograph. Vasodilation will be determined by micrometer measurements derived from small steel wires inserted into the aortic ring lumen.	3-4		66% (Defined experimental parameters for the scratch wound assay and macrophage chemotaxis assay, but not the aortic ring assay).
Subtask 2. Test 7 candidate partial agonist compounds in wound healing assays using the SVEC4-10 mouse endothelial cell line. These compounds are commercially available (no MTA required).	3-6		50% (Tested 3 candidate partial agonists in the scratch assay)
Subtask 3. Test 7 candidate partial agonist compounds in chemotaxis assays with macrophages from WT C57BL/6 mice and NOPR-deficient mice, the latter to confirm specificity	5-12	13 (Confirmed the lack of agonist action of Ro-64-6198 in chemotaxis)	
Subtask 4. Test 7 candidate partial agonist compounds in aortic ring vasodilation assays using tissues from WT C57BL/6 and NOPR-deficient mice, the latter to confirm specificity	5-12		20% (successfully revived $NOPR^{+/-}$ mice from cryopreservation)

Milestone(s): Select one partial agonist compound based on its wound healing, chemotaxis, and vasodilation profile for in vivo testing. The criteria for compound selection will be based on the comparative performance of compounds in the in vitro assays. The compound we seek will have strong inhibitory effects on macrophage migration, strong activating effects in wound healing and vasodilation, and its effects will be NOPR-specific. Of the 7 compounds we will screen in the in vitro assays, we will select the partial agonist that best embodies these properties.			66% (Compounds 12 and 7 slightly better than compound 8 when used at 100 μM in wound healing; unable to evaluate effects on macrophage chemotaxis due to lack of agonist activity of Ro64-6198)
Specific Aim 2 **To test the hypothesis that NOPR partial agonists prevent and reverse scleroderma pathogenesis.**			
Major Task 3: Test the hypothesis that NOPR partial agonists ameliorate scleroderma pathogenesis in vivo	13-18		90%
Subtask 1. Perform 1-2 in-life bleomycin-induced scleroderma studies using C57BLl/6 and NOPR-/- mice treated prophylactically (treatment start on day 1) or therapeutically (treatment start on Day 15) with NOPR agonist (N/OFQ), antagonist (JTC-801), or partial agonist to be determined from in vitro studies in Major Task 2.	13-16	18	
Subtask 2. Perform histological evaluation of tincture-stained skin study samples (H&E for general skin architecture and leukocyte infiltrates, trichrome for fibrosis evaluation).	15-18	18	
Subtask 3. Perform immunoassay evaluation of scleroderma pathology biomarkers in skin study samples.	15-18		10% -we have archived samples for future analysis

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the

project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

Major Activity 1:
Administrative tasks and experimental preparation
Specific Objectives:
Subtask 1. Obtain local IACUC and ACURO approval for the animal studies.
Subtask 2. Obtain and expand SVEC4-10 mouse cell line (ATCC).
Subtask 3. Obtain NOPR-/- animals and establish breeding colony
Results and Conclusions:
1) We obtained the necessary local IACUC and ACURO approval for the proposed animal studies.
2) We obtained and expanded the endothelial cell line SVEC4-10 from the ATCC.
3) We obtained 2 $NOPR^{+/-}$ animals and confirmed the genotype by in-house PCR (**Fig. 1**). We successfully crossed $NOPR^{+/-}$ x $NOPR^{+/-}$ mice and generated $NOPR^{-/-}$ mice at the anticipated Mendelian frequency. We successfully crossed $NOPR^{-/-}$ x $NOPR^{-/-}$ mice and established a small breeding colony of $NOPR^{-/-}$ mice.

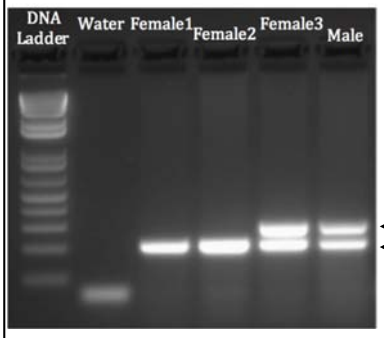


Figure 1. Successful resuscitation of $NOPR^{-/+}$ mice from cryopreserved sperm by the Mutant Mouse Regional Resource Center (MMRRC). The MMRRC was successful on its second attempt at resuscitating $NOPR^{-/+}$ mice from cryopreserved sperm. We received four mice, two of which (one male and one female) were confirmed by our in-house PCR genotyping analysis to be $NOPR^{-/+}$. We have since mated the heterozygous founders with WT C57bl/6 mice to generate additional $NOPR^{-/+}$ mice. We intercrossed the $NOPR^{-/+}$ to generate $NOPR^{-/-}$ mice.

Stated Goals Not Met: None.

Major Activity 2:
To test NOPR partial agonists for activity in three in vitro assays: endothelial cell wound healing, macrophage chemotaxis, and blood vessel tension (aortic ring assay)
Specific Objectives:
Subtask 1. Determine optimal NOPR agonist (N/OFQ) and antagonist (JTC-801) concentrations for the three proposed in vitro assays: scratch wound assay, macrophage chemotaxis using transwell inserts, and blood vessel tension using an aortic ring assay. In the scratch wound assay, a pipet tip is used to scratch or wound an endothelial monolayer, and the 'time-to-wound closure' is monitored over the next 24 hr by microscopy. In the macrophage chemotaxis assay, cells are loaded into the top well of a transwell insert and exposed to chemoattractants in the bottom well; the number of migrating cells is quantified by flow cytometry. In the aortic ring assay, freshly isolated thoracic aorta rings will be harvested and mounted in a small-vessel myograph. Vasodilation will be determined by micrometer measurements derived from small steel wires inserted into the aortic ring lumen.

Subtask 2. Test 7 candidate partial agonist compounds in wound healing assays using the SVEC4-10 mouse endothelial cell line. These compounds are commercially available (no MTA required).
Subtask 3. Test 7 candidate partial agonist compounds in chemotaxis assays with macrophages from WT C57BL/6 mice and NOPR-deficient mice, the latter to confirm specificity
Subtask 4. Test 7 candidate partial agonist compounds in aortic ring vasodilation assays using tissues from WT C57BL/6 and NOPR-deficient mice, the latter to confirm specificity

Results and Conclusions

1) We successfully procured NOPR agonist Ro-64-6198, antagonist SB612111, and three partial agonists (compounds 7, 8, and 12) (**Revised Table 1**). For our initial studies, we elected to focus on evaluating a single NOPR agonist (Ro-64-6198), a single antagonist (SB612111), and three partial agonists, rather than N/OFQ agonist, J113397 and JTC-801 antagonists, and the four other proposed partial agonists to conserve resources. We selected Ro64-6198 over NOPR peptide ligand N/OFQ because Ro64-6198 uses the same formulation vehicle (DMSO) as the test articles, thus reducing the number of vehicle controls needed for each experiment and making comparisons among compounds more accurate and independent of possible vehicle effects. We selected antagonist SB612111 based on its favorable Ki compared with the other two antagonists. We selected partial agonists 7, 8, and 12 based on their Ki and GTPγS profiles, with the goal of spanning the range of binding affinities and GTPγS stimulation. Compound 7 has high binding affinity and good GTPγS stimulation; compound 8 has lower binding affinity and weaker GTPγS stimulation capacity; compound 12 has middle-of-the-road properties (**Revised Table 1**).

Revised Table 1. Receptor binding affinities K_i and GTPγS binding (EC_{50} and % stimulation) to NOPR of procured compounds.

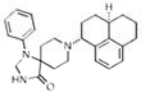
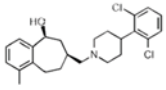
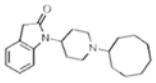
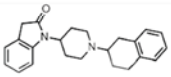
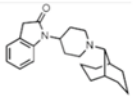
Compound	Structure	K_i (nM)	EC_{50} (nM) [% stim.]	Quantity (mg)	Cost ($)	Note
2 Ro 64-6198		0.39	38 [100]	750	6,500	NOPR agonist
4 SB612111		1.42±0.12	NA	200	2,770	NOPR antagonist
7 (WuXi 1c)		1.39±0.42	19.9±3.4 [59.1±7.1]	750	1,600	test compound
8 (WuXi 1d)		29.3±11.4	92.5±16 [30.9±3.1]	616	1,600	test compound
12 (WuXi 1m)		7.49±0.78	28.7±0.6 [45±5]	668	1,600	test compound

Table shows K_i and EC_{50} from different publications[6,7]. EC_{50} values are not available for antagonists.

2) We successfully established the in vitro endothelial cell scratch assay (**Fig. 2A**), and evaluated Ro64-6198, SB612111, 7, 8, and 12 for activity. In preliminary studies, the two partial agonists 8 and 12, as well as antagonist SB612111 improved wound closure compared with vehicle alone (0.1% DMSO) or NOPR agonist Ro-64-6198 when tested at 1 μM (**Fig. 2B**). At 100 uM, all three partial agonists and SB612111 improved wound closure compared with vehicle or Ro-64-6198 (**Fig. 2C**). Compounds 7 and 12 showed a dose-dependent response in improving wound closure (**Fig. 2B,C**). Thus, partial agonist 12 has the most promising wound closing properties in the SVEC4-10 endothelial cell scratch assay.

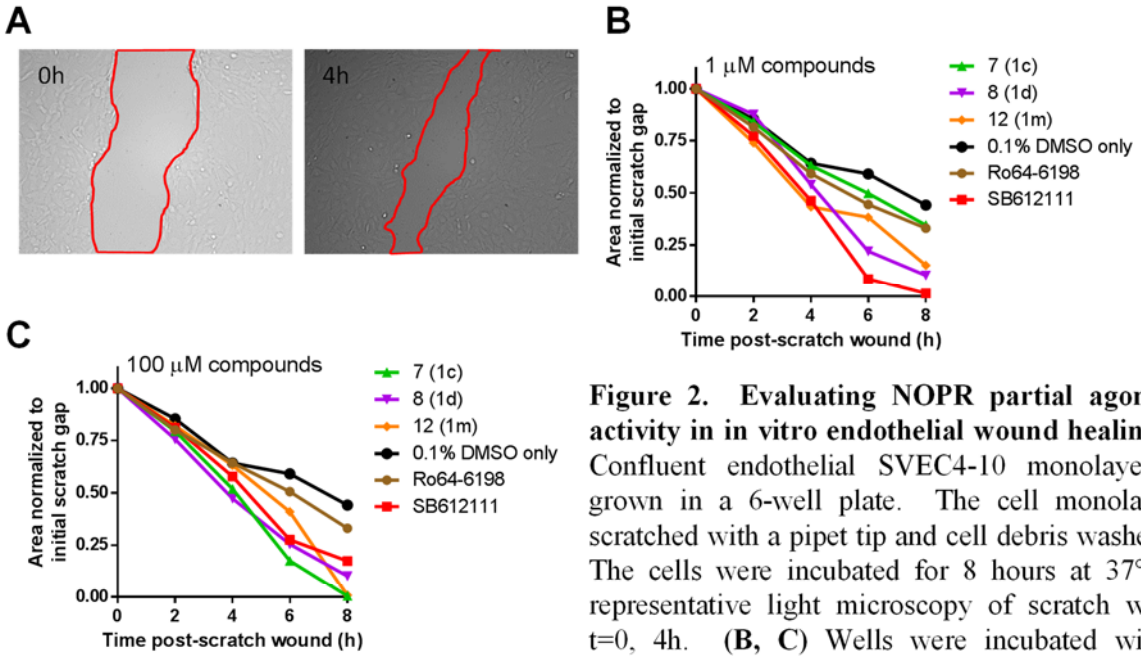


Figure 2. Evaluating NOPR partial agonists for activity in in vitro endothelial wound healing assay. Confluent endothelial SVEC4-10 monolayers were grown in a 6-well plate. The cell monolayer was scratched with a pipet tip and cell debris washed away. The cells were incubated for 8 hours at 37°C. **(A)** representative light microscopy of scratch wound at t=0, 4h. **(B, C)** Wells were incubated with three different NOPR partial agonists (1 μM, **B**; or 100 μM, **C**) or 0.1% DMSO (vehicle control), and the plate was photographed every 2 hours. ImageJ was used to quantify the scratch area over time. Data presented normalized to the initial area. N=1 experiment, one well per compound.

3) We successfully established in vitro transwell chemotaxis of resident peritoneal mouse leukocytes (**Fig. 3A**), thioglycollate-elicited mouse peritoneal monocyte/macrophages (**Fig. 3B**), in vivo GM-CSF-expanded bone marrow myeloid precursors (**Fig. 3C**), and human blood CD14+ monocytes (**Fig. 3D**) to positive control chemokine CXCL12 (100 nM). Migration of these otherwise motile cells to various concentrations of NOPR agonist Ro64-6198 was not above background levels (assessed as cell migration to no chemoattractant) (**Fig. 3**), even though Ro64-6198 was reported by others to induce mouse macrophage migration. Thus, based on our well-controlled in vitro chemotaxis studies, mouse myeloid cell precursors present in bone marrow, resident tissue mouse macrophages, inflammatory-stimuli-elicited mouse monocytes, and human blood monocytes do not migrate to NOPR agonist Ro64-6198.

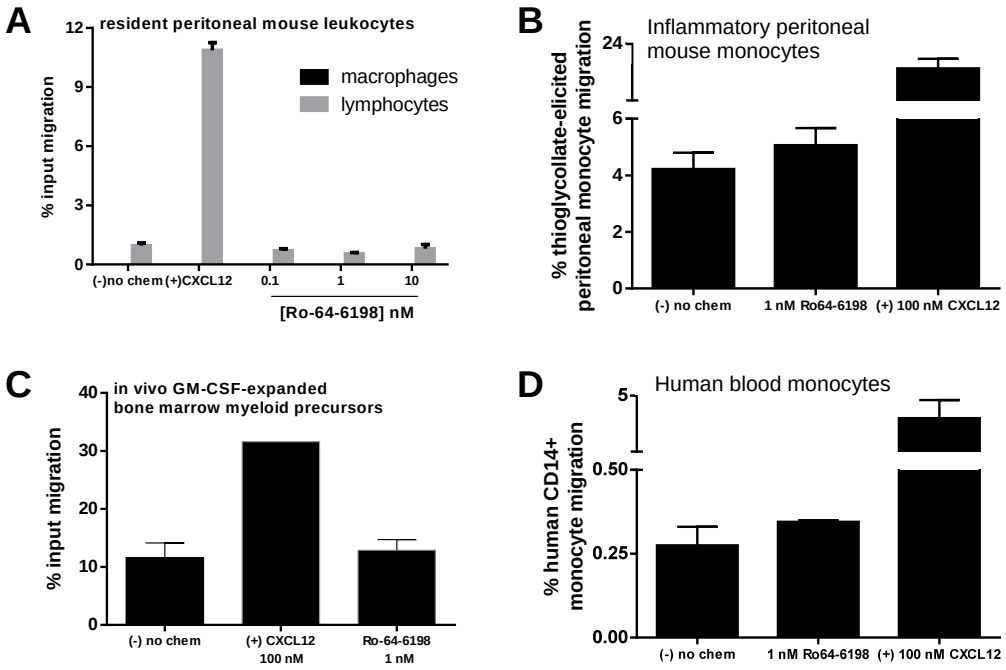


Figure 3. Testing NOPR agonist Ro-64-6198 for chemotactic activity using mouse and human monocytes/macrophages. One million resident mouse peritoneal mononuclear cells (**A**), thioglycollate-elicited mouse peritoneal monocytes (**B**), GM-CSF-expanded bone marrow myeloid cell precursors (**C**), or human peripheral blood mononuclear cells (**D**) were added to the top well in transwell filter plates. The indicated concentrations of NOPR agonist Ro64-6198 was added to the bottom chamber, and after 2 h incubation at 37°C the cells that migrated to the bottom well were quantified by flow cytometry. For human PBMC, the cells were stained for monocyte marker CD14 prior to quantification. Negative control: (-) no chemoattractant. Positive control: (+) 100 nM CXCL12 (CXCR4 agonist). Mean ± range, duplicate wells (A, B, D) or mean ± SEM (C, except the (+) control, which was a single well).

Stated Goals Not Met:

We did not test the NOPR compounds in the aortic ring assay (target date was month 12). The local lab (Phil Tsao, PI) we had previously contacted to assist with this approach was unable to supply the organ culture apparatus or technical expertise needed for the assay. We tested 3 representative partial agonists (of the 7 proposed) in the wound healing assay by month 8 (instead of month 6). We were delayed due to the failure of our initial commercial chemistry company (Proactive Molecular Research) to deliver compounds 7, 8, and 12 (they were unable to complete the synthesis with the desired purity). The second company we selected (WuXi) was able to provide adequate quantities of the compounds with the necessary purity. As mentioned above, we were unable to establish an adequate chemotactic window (monocyte/macrophage migration to NOPR agonist Ro64-6198) to enable testing our partial agonist compounds for inhibitory activity as proposed (the target for this was month 12).

Major Activity 3:

Test the hypothesis that NOPR partial agonists ameliorate scleroderma pathogenesis in vivo

Specific Objectives:

Subtask 1. Perform 1-2 in-life bleomycin-induced scleroderma studies using C57Bl/6 and NOPR$^{-/-}$ mice treated prophylactically (treatment start on day 1) or therapeutically (treatment start on Day 15) with NOPR agonist (N/OFQ), antagonist (JTC-801), or partial agonist to be determined from in vitro studies in Major Task 2.
Subtask 2. Perform histological evaluation of tincture-stained skin study samples (H&E for general skin architecture and leukocyte infiltrates, trichrome for fibrosis evaluation).
Subtask 3. Perform immunoassay evaluation of scleroderma pathology biomarkers in skin study samples.

Results and Conclusions:

1) We performed a pilot study using bleomycin to induce experimental scleroderma in C57bl/6 mice. Twenty-eight days after the initial s.c. bleomycin injections we euthanized the mice and evaluated the skin by histology. Bleomycin induced significant dermal skin thickening in a dose-dependent manner, with homogeneous deposits visible by H&E staining that stained light blue upon Masson trichrome stain, indicating collagen deposition/fibrosis (**Fig. 4**). Thus, we successfully established the in vivo scleroderma in our lab for evaluation of NOPR compounds for efficacy and effects on disease pathogenesis.

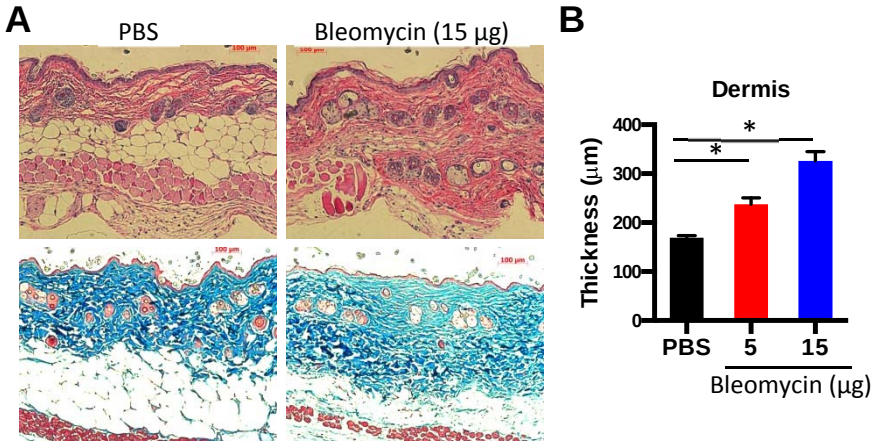


Figure 4. Local bleomycin injections induce clinical and histological scleroderma-like disease including dermal thickening and collagen deposition. C57B6 mice were injected with 100µl PBS or bleomycin (dissolved in PBS at two different concentrations; 50µg/ml or 150µg/ml) subcutaneously into the shaved back 5 times/week for 4 weeks. One day following the final injection, lesional skin was removed, fixed in 10% formalin, embedded in paraffin and sections were stained with either hematoxylin and eosin (H&E) (**A**, *top panels*) or Mason trichrome stain. (**A,** *bottom panels***).** Representative images of the skin stained for H&E or Mason trichrome, 20X magnification. Note the dense collagen network (light blue trichrome stain) and dermal thickening in the bleomycin-treated skin compared with controls. ***(B)*** Dermal thickness defined as the distance between the epidermal-dermal junction and the dermal-adipose layer junction was determined in the H&E-stained sections. Mean ± SEM, 3 mice/group (8 measurements/section, 2 sections/mouse were averaged), *p<0.05 by t-test.

2) We performed 2 in-life bleomycin-induced scleroderma studies using WT C57Bl/6 mice treated prophylactically (treatment start on day 1) or therapeutically (treatment start on Day 15) with NOPR agonist Ro64-6198, antagonist SB612111, or two NOPR partial agonists: #7 and #12. Subcutaneous bleomycin induced a significant increase in epidermal and dermal skin thickness compared with PBS (332 ± 35 µm vs. 143 ± 9 µm, respectively, mean ±SEM) (**Fig. 5**).

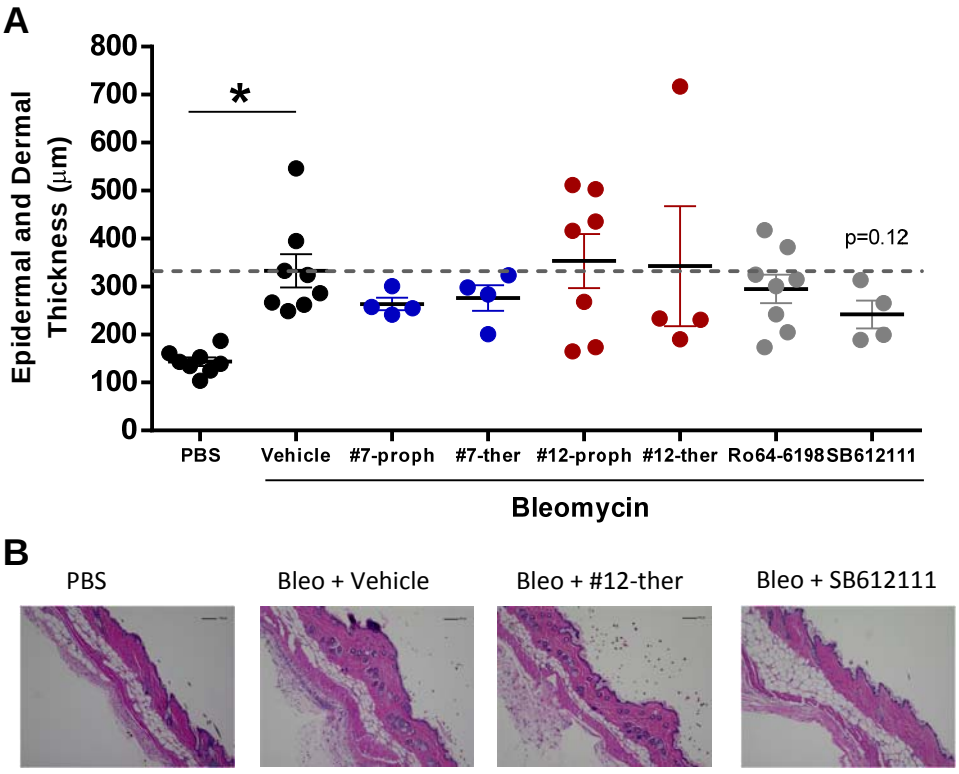


Figure 5. Evaluation of NOPR agonist Ro64-6198, antagonist SB612111, and partial agonists #7 and #12 in experimental bleomycin-induced skin scleroderma. C57B6 mice were injected with 100ul PBS or bleomycin (dissolved in PBS 50ug/ml) subcutaneously into the shaved back 5 times/week for 4 weeks. Three times/week, Ro64-6198, SB612111, or partial agonist compounds #7 and #12 (dissolved in corn oil vehicle at 1mg/ml) were administered by intraperitoneal injection (200ul; 10mg/kg) starting on Day 1 of bleomycin injection (prophylactic treatment regimen, #7-proph, #12-proph). We also evaluated compounds #7 and #12 for efficacy as a treatment for established scleroderma, beginning treatment 15 days post-bleomycin injection (treatment regimen, #7-ther, #12-ther). One day following the final injection, lesional skin was removed, fixed in 10% formalin, embedded in paraffin and sections were stained with hematoxylin and eosin (H&E). **(A)** Epidermal and dermal thickness, defined as the distance from the outer epidermis to the dermal-adipose layer junction, was quantified in the H&E-stained sections. Mean ± SEM, 4-8 mice/group (10 measurements/section) combined from 2 independent experiments, *$p<0.05$ by ANOVA with Bonferroni post-hoc test; exact p-value for SB612111 vs. Vehicle (all bleomycin treated) included (t-test, 2-tailed). **(B)** Representative images of the skin stained for H&E, 10X magnification. Note the significant dermal thickening in the bleomycin-treated skin compared with controls.

Prophylactic or therapeutic treatment with compound #7 or #12 (i.p. 3x/week, 10 mg/kg in corn oil) did not impact bleomycin-induced skin fibrosis as quantified by histological measurements of epidermal and dermal thickness (**Fig. 5**). Prophylactic treatment with NOPR agonist Ro64-6198 did not impact disease progression (**Fig. 5**). Prophylactic treatment with NOPR antagonist SB612111 reduced skin fibrosis induced by bleomycin by approx. 50% (epidermal and dermal thickness: 242 ± 21 µm, mean ±SEM), although this did not reach statistical significance (p=0.12, 2-tailed t-test) (**Fig. 5**). We conclude that while NOPR agonists and partial agonists did not impact bleomycin-induced skin fibrosis at the doses tested, there was a trend that NOPR antagonist SB612111 suppressed experimental dermal fibrosis.

3) We evaluated the effects of NOPR-deficiency on experimental skin scleroderma. NOPR deficiency significantly inhibited bleomycin-induced skin fibrosis. While bleomycin induced a 3-fold increase in epidermal and dermal skin thickness in WT mice (PBS vs. bleomycin, 141 ± 20 µm vs. 438 ± 84 µm, mean ±SEM), NOPR deficiency protected against experimental scleroderma (PBS vs. bleomycin, 176 ± 24 µm vs. 220 ± 7 µ, mean ±SEM) (**Fig. 6**). This results somewhat aligns with the effect of NOPR antagonist SB612111 in **Fig. 5**. We conclude that NOPR plays a pathogenic role in experimental skin scleroderma, and that small molecule NOPR antagonists may prove therapeutic in a clinical setting.

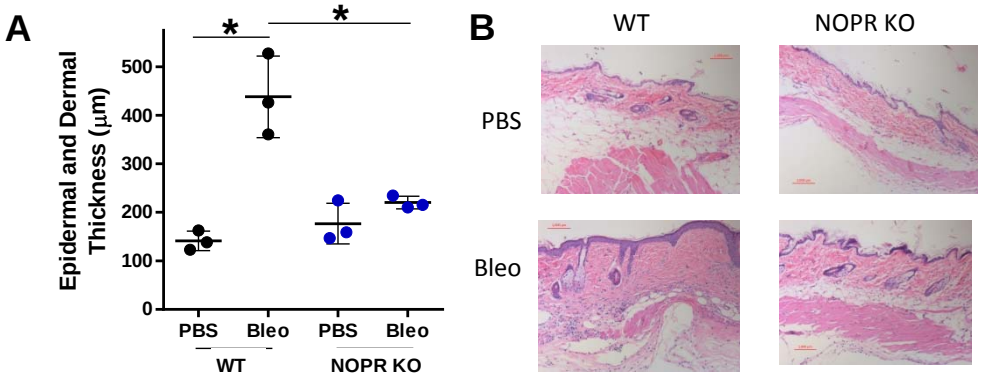


Figure 6. NOPR deficiency protects against bleomycin-induced clinical and histological scleroderma-like disease. WT C57B6 or NOPR-deficient mice were injected with 100ul PBS or bleomycin (dissolved in PBS at 50ug/ml) subcutaneously into the shaved back 5 times/week for 4 weeks. One day following the final injection, lesional skin was removed, fixed in 10% formalin, embedded in paraffin and sections were stained with hematoxylin and eosin (H&E). (**A**) Epidermal and dermal thickness, defined as the distance from the outer epidermis to the dermal-adipose layer junction, was quantified in the H&E-stained sections. Mean ± SEM, 3 mice/group (10 measurements/section), *$p<0.05$ by ANOVA with Bonferroni post-hoc test. **(B)** Representative images of the skin stained for H&E, 20X magnification. Note the dermal thickening in the bleomycin-treated skin compared with PBS controls, particularly in the WT mice.

Stated Goals Not Met:

In subtask 3 we proposed to perform immunoassay evaluation of scleroderma pathology biomarkers in skin study samples. We have archived skin material from compound treated and NOPR$^{-/-}$ mice for future analysis. We have paraffin-embedded skin samples, unstained paraffin sections, skin tissue frozen in liquid nitrogen, and skin tissue frozen in OTC. Due to time and financial constraints, we were unable to process the samples by the end of the project. However, as we move forward with our manuscript describing these results, we will analyze the tissues as needed for the publication.

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Dr. Melissa LaJevic was the project lead for experiments performed supporting the Aims of this grant. She mentored Dr. Nicole Salazar in using bleomycin to induce skin fibrosis in mice. Dr. LaJevic and Dr. Zabel worked one-on-one to design the in vivo experiments, and Dr. Zabel was able to provide insight into key parameters involved in drug dosing and outcome measurements. Dr. LaJevic and Dr. Salazar gained greater proficiency in in vivo skin disease models and novel drug testing.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Nothing to Report, although we have a manuscript in preparation and plan on discussing the data at the Montagna Symposium on the Biology of the Skin, October 2016 (Dr. LaJevic's conference registration is supported by the National Psoriasis Foundation).

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

Scleroderma is a debilitating connective tissue disease that affects approximately 100,000 people in the United States with no known cure and few effective treatments. Here we discovered that serpentine neuropeptide receptor NOPR plays a pathogenic role in experimentally-induced scleroderma in mice. Genetic deficiency in NOPR or treatment with pharmaceutical agents that inhibit NOPR substantially reduced histological features of skin fibrosis in mouse scleroderma models. These results suggest that NOPR is a therapeutic target in scleroderma, and will likely stimulate research into defining the cellular and molecular mechanisms by which NOPR and its endogenous neuropeptide ligand contribute to skin fibrosis.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

This project introduces a novel role for NOPR in skin fibrosis, which may extend to other fibrotic diseases such as pulmonary or liver fibrosis. Our results may have a particularly significant impact in the study of lung fibrosis, since the use of bleomycin (Blenoxane) as a front line chemotherapeutic agent for various types of cancer can lead specifically to lung fibrosis in patients. NOPR antagonists may prove to be a beneficial countermeasure to bleomycin-induced lung fibrosis.

What was the impact on technology transfer?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

We will submit an invention disclosure to the VA Technology Transfer Office and the Stanford Office of Technology Licensing on the potential use of targeting NOPR genetically or pharmaceutically to suppress skin scleroderma. Commercial entities may seek to license this technology for clinical development.

What was the impact on society beyond science and technology?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to Report

5. **CHANGES/PROBLEMS:** The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

Changes in approach and reasons for change

Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Nothing to Report

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Obtaining the NOPR-targeted mice and NOPR small molecules was delayed due to issues with the commercial entities supplying these materials. This was discussed in more detail in section 3 above. Our collaborators on the in vitro aortic ring assay were unable to provide the necessary equipment and expertise to aid in testing the NOPR compounds for effects on vessel contraction. Moving forward, although this project has come to an end, we have identified a backup lab to assist with this assay and plan on pursuing the experiments described.

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to Report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Nothing to Report

Significant changes in use or care of vertebrate animals.

Nothing to Report

Significant changes in use of biohazards and/or select agents

Nothing to Report

6. PRODUCTS: List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted,*

awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).

Nothing to Report—a manuscript, invention disclosure, and conference abstract/presentation are under preparation.

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: Author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to Report

Other publications, conference papers, and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Nothing to Report

- **Website(s) or other Internet site(s)**

List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to Report

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. In addition to a description of the technologies or techniques, describe how they will be shared.

Nothing to Report

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. State whether an application is provisional or non-provisional and indicate the application number. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to Report, an invention disclosure is under preparation.

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment, and/or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:
  - *data or databases;*

- *biospecimen collections;*
- *audio or video products;*
- *software;*
- *models;*
- *educational aids or curricula;*
- *instruments or equipment;*
- *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
- *clinical interventions;*
- *new business creation; and*
- *other.*

Live NOPR-deficient mice were generated for use as research material. Small molecule NOPR partial agonists were generated for use as research material.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change."

Name:	Brian Zabel
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	ZABEL.BRIAN (eRA Commons ID)
Nearest person month worked:	3
Contribution to Project:	Dr. Zabel has overseen all aspects of the project. He successfully sourced the NOPR small molecules (**Revised Table I**), managed the resuscitation and delivery of NOPR KO mice, designed in vivo and in vitro experiments (**Figs. 1-6**), and analyzed results.
Name:	Nicole Salazar
Project Role:	Research Assistant
Researcher Identifier (e.g. ORCID ID):	nsala001 (eRA Commons ID)
Nearest person month worked:	0
Contribution to Project:	Dr. Salazar assisted with the in vitro wound closing assay (**Fig. 2**) and genotyping the NOPR KO mice (**Fig. 1**).

Name:	Melissa LaJevic
Project Role:	Research Assistant
Researcher Identifier (e.g. ORCID ID):	LAJEVIC.MELISSA (eRA Commons ID)
Nearest person month worked:	6
Contribution to Project:	Dr. LaJevic performed all of the in vivo experimental scleroderma studies and prepared and evaluated and quantified histological changes in the affected skin (**Fig. 4,5,6**). She also performed in vitro chemotaxis experiments (**Fig. 3**).

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

ZAB0001APR **7/1/16-6/30/17**
National Psoriasis Foundation
Anti-Inflammatory Chemerin C15 Peptide for The Topical Treatment of Psoriasis
This is a new award.

ZAB0001ARG **1/1/15-9/30/15**
Rogne Bioscience, Inc.
Role of Chemerin Peptides in Psoriasis
This project has ended.

ZAB0002AOM **8/1/16-7/31/17**
Stanford Office of Technology and Licensing / Stanford Innovator Program
Small Molecule CMKLR1 Antagonists in Demyelinating Disease
This is a new award.

ECCRF01 (Zabel, Co-I) **7/1/16-6/30/18**
Emerson Collective Cancer Research Fund
CCRL2 Antagonists for Cancer Therapy
This is a new award.

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.
Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating PI and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to **https://ers.amedd.army.mil** for each unique award.

QUAD CHARTS: If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.

9. **APPENDICES:** Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

Targeting the Nociceptin/Orphanin FQ Receptor for Scleroderma Therapy

PR131037
W81XWH-15-1-0001

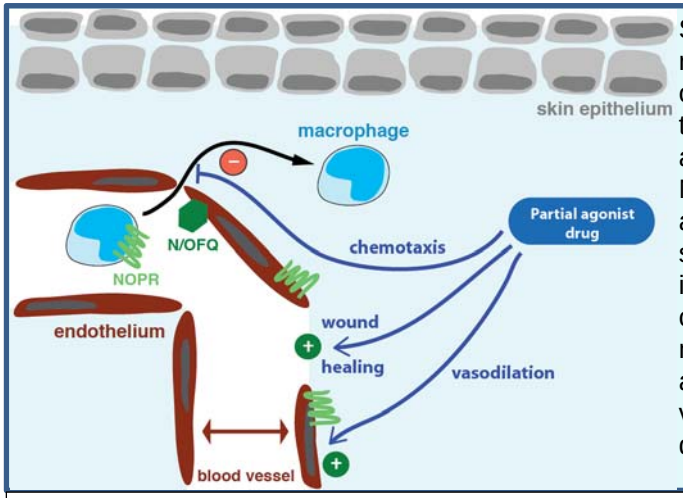
PI: Dr. Brian Zabel **Org:** Palo Alto Veterans Institute for Research **Award Amount:** $175K

Study/Product Aim(s)

- Test the hypothesis that distinct partial agonists for the Nociceptin/Orphanin FQ receptor (NOPR) limit macrophage migration and vasculopathy
  - Test the hypothesis that N/OFQ increases macrophage recruitment into inflamed sites via expression of NOPR
  - Test the hypothesis that NOPR partial agonists counter scleroderma vasculopathy
- Test the hypothesis that NOPR partial agonists ameliorate scleroderma pathogenesis

Approach

The effect of small molecule drug candidates will be tested in macrophage chemotaxis, endothelial wound healing and vasodilation assays *in vitro*. Drug candidate(s) will be selected and tested in a bleomycin-induced scleroderma model *in vivo*.



Schematic representation of our hypothesis that partial agonist drugs for NOPR ameliorate scleroderma by inhibiting chemotaxis of macrophages and limiting vascular dysfunction

Activities: Generated NOPR KO mice. Performed in vitro wound healing and macrophage chemotaxis studies. Performed in vivo bleomycin-induced scleroderma studies evaluating effects of NOPR compounds and NOPR genetic deficiency.

Timeline and Cost

Activities CY	14	15	16	
NOPR *in vitro* assays Milestone: Select 1-2 compounds				
NOPR scleroderma *in vivo* study Milestone: Submit manuscript				
Estimated Budget ($K)	**$19**	**$110**	**$46**	

Updated: 8/3/2016

Goals/Milestones (Example)

FY15 Goal – NOPR *in vitro* assays
☑Obtain ACURO approval for animal studies
☑ Obtain NOPR$^{-/+}$ animals, start breeding colony
☑ Obtain and expand SVEC4-10 mouse cell line
☑ Perform chemotaxis assays
☑ Perform wound healing assays
☐Perform vasodilation assays
☑ Select 1–2 compounds based on their *in vitro* profile

FY16 Goals – NOPR scleroderma *in vivo* studies
☑Establish preclinical bleomycin-induced scleroderma model
☑Test compounds for efficacy in experimental scleroderma
☑Evaluate experimental scleroderma in NOPR-deficient mice
☐Submit manuscript for publication

Comments/Challenges/Issues/Concerns

- Unanticipated delays in obtaining NOPR$^{-/-}$ mice and NOPR compounds

Budget Expenditure to Date

Projected Expenditure: $175,000.00
Actual Expenditure: $174,945.27